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(71) Applicant *(for all designated States except US)*: ARQULE, INC. [US/US]; Suite 1000, 200 Boston Avenue, Medford, MA 02155 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LI, Yu-Tysr [CN/US]; Suite 1000, 200 Boston Avenue, Medford, MA 02155 (US). KYRANOS, James, N. [US/US]; Suite 1000, 200 Boston Avenue, Medford, MA 02155 (US).

(74) Agent: KINDREGAN, Helen, C.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).

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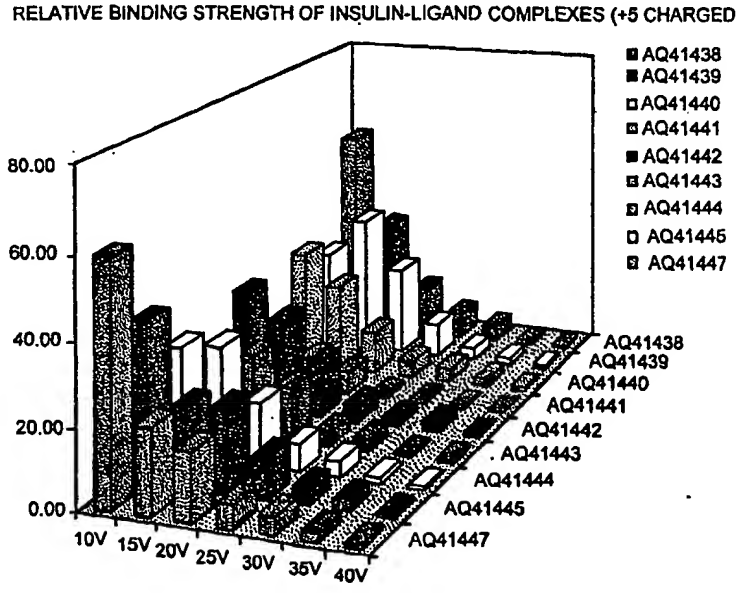
(54) Title: AN AUTOMATED, HIGH THROUGHPUT METHOD FOR SCREENING A PLURALITY OF COMPOUNDS USING MASS SPECTROMETRY

(57) Abstract

The present invention is directed to a method for the rapid testing of a plurality of compounds for non-covalent interaction with at least one target. The method includes the steps of providing a continuous flow of a mobile phase into a mass spectrometer; providing settings for the mass spectrometer for detecting at least one target in the mobile phase; sequentially injecting individual samples of a plurality of mixtures, wherein each mixture comprises the at least one target and at least one compound of interest, into the mobile phase for delivery into the mass spectrometer; and obtaining a mass spectrum that indicates for each mixture the presence or absence of a complex of a target and a compound of interest. Preferably, each sample is completely injected into the mobile phase within less than 5 minutes, and more preferably within less than 1 minute and even more preferably within less than 30 seconds. The method of the invention may be multiplexed by forming a mixture containing one target and a plurality of compounds of interest; one compound of interest and a plurality of targets of receptor proteins or receptor-ligand complexes. The plurality of mixtures are typically formed in a plurality of addressable locations, such as the wells of a multiple-well plate. Samples of the mixtures are then obtained from the addressable locations, and sequentially injected into the mobile phase for delivery to the mass spectrometer. The relative strength of the interaction between the target and the compound of interest within each complex also may be determined by dissociating at least one complex of a target and a compound of interest.



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AN AUTOMATED, HIGH THROUGHPUT METHOD FOR SCREENING A PLURALITY OF COMPOUNDS USING MASS SPECTROMETRY

Field of the Invention

The present invention relates to an automated, high-throughput method of screening a plurality of chemical compounds to detect interaction of those compounds with a target using mass spectrometry ("MS") or tandem mass spectrometry ("MS/MS") and/or to determine the affinity of the binding interaction.

Background of the Invention

Molecular recognition events are a hallmark of biological systems. For example, particular enzymes interact with specific substrates, histones specifically bind to DNA, major histocompatibility complexes are specifically recognized by T cell receptors, and immunoglobulins exhibit extremely specific binding to certain antigens. Researchers in the life sciences seek to understand and exploit these molecular recognition events within a specific biological system to discover methods to manipulate the system to obtain a desired result. The paradigm approach is the use of a chemical compound (a drug) to inhibit binding of a naturally occurring ligand to a receptor in order to achieve a desired therapeutic effect. This use of molecular recognition events to manipulate biological systems with chemical compounds is used by the agrochemical industry (e.g., herbicides, fungicides, insecticides) as well as the pharmaceutical and biotechnological industries. In addition, one can exploit a specific molecular recognition event between a target and ligand as a means of separating the target from a complex mixture by attaching the ligand to a stationary phase, combining the mixture in a mobile phase (e.g., solvent or gas) with the ligand in a stationary phase under conditions in which the ligand and target will bind, eluting the undesired elements from the mixture, and then eluting the target from the ligand. This method is commonly referred to as affinity chromatography.

The conventional process used to detect these molecular recognition events between a target and chemical compound relies on biological screens to detect the effect of the compound on the biological system, or subset of the biological system. For example, in order to detect the effect of a chemical compound on a receptor, one would ordinarily develop a screen involving the receptor, its natural ligand, and a reporter element (such as a fluorescent compound) that is activated in proportion to activation of the receptor. If the compound of interest inhibits the receptor-ligand interaction, the screen would quantitatively detect the inhibition effect. Although

this approach enjoys nearly universal acceptance, it has at least two important drawbacks.

First, these biological screening methods are typically expensive and time-consuming to perform on very large numbers of compounds. Although the per unit cost of a screen for a compound has declined with the widespread use of high-throughput screening methods, which make extensive use of robots and automation, the numbers of compounds that require testing have increased dramatically with the advent of combinatorial chemistry and combinatorial biology technology, as well as enhanced efforts to increase natural products sources (e.g., organisms from volcanic vents on the ocean floor). This increase in compound numbers means that the per target screening costs may remain constant or increase despite the substantial investment in high-throughput screening equipment.

Second, assay development and validation is usually an expensive and time-consuming process, even with high-throughput screening methods. Assay development usually requires extensive knowledge of the particular biological system. Typically, a new screening method must be developed and validated for each biological target, although similar methods may be used with similar targets. More recently, the advent of genomics technology has enabled researchers to link biological targets with disease states without knowledge of target function or the biological system in which the target participates. In order to use conventional biological assays with such uncharacterized targets, one must identify the biological function of the target with little information to provide useful guidance. This problem exacerbates the time and expense required for assay development and validation.

Because of these problems with use of biological assays to detect molecular recognition events between biological targets and chemical compounds, life sciences researchers would benefit from the development of a method to detect such interactions at a lower per unit cost and without extensive target-specific assay development.

In the early 1990s, biomedically interesting interactions, such as enzyme-substrate, receptor-ligand, peptide-peptide, metal-protein, DNA duplex, etc., were detected by electrospray MS via continuous infusion using a syringe infusion pump. Electrospray and related atmospheric pressure ionization (API) techniques such as ionspray (pneumatically assisted electrospray) and atmospheric pressure chemical ionization (ApcI) form gas phase ions directly from solution at atmospheric pressure via protonation (or deprotonation in the negative ion mode) followed by ion evaporation. The atmospheric ionization techniques not only ionize small molecular weight compounds, but large macromolecules as well. In fact, these ionization

techniques can also provide a multiple number of charges on macromolecules capable of stabilizing them. Since all mass analyzers separate ions based on their mass-to-charge ratio (m/z), the greater the number of charges stabilized on the macromolecule, the lower the mass-to-charge ratio, so that high molecular weight compounds can be observed with a quadrupole mass spectrometer operated under unit mass resolution. Moreover, because of the ionization conditions typical of electrospray operation (and other API techniques), fragmentation of covalent bonds is typically not observed.

Typically, a continuous infusion electrospray MS interface is set up using a syringe infusion pump to continuously infuse solution mixtures containing a specific target, such as a macromolecule, and a molecule of interest, such as a potential ligand, into a mass spectrometer. The syringe is usually filled with the solution mixture and attached to the infusion pump. A small diameter capillary is attached from the syringe needle directly to the particular API interface of the mass spectrometer, and infusion of the solution begins. The normal infusion flow rate is slow, and typically ranges from about 2 μl/minute to about 20 μl/minute. The mixtures are sprayed, evaporated, and ionized with the aid of a pneumatically-assisted nebulizing gas while continually flowing into the mass spectrometer. The gas phase ions, including the targets, the molecules of interest, and any complexes formed therefrom are detected and identified by the mass spectrometer.

The continuous infusion method has been used with biologically relevant macromolecules because such targets require an aqueous solution in order to maintain the established solution equilibrium of the target-ligand complex under physiological conditions. The aqueous solution, however, causes an unfavorable signal-to-noise ratio. In order to prevent an unfavorable signal-to-noise ratio, continuous infusion of the mixture during the MS experiment (usually 10-15 minutes cycle time between different samples) is used so that the data may be acquired using the multiple channel average (MCA) mode of the mass spectrometer. The MCA mode allows the individual scans to be continuously accumulated over a period of time. This accumulation of data enhances a relatively weak signal of a target-ligand complex above the random chemical background noise originating from the aqueous medium. The accumulated MCA scan provides better sensitivity, and a superior signal to noise ratio, than any single discrete scan or average number of scans. This method, however, requires a sample analysis time on the order of greater than ten minutes.

Summary of the Invention

The present invention is based on the finding that non-covalent binding interactions can be detected using a high-throughput, automated screening method that is more efficient than conventional high-throughput biological screening methods (including both assay development costs and screening costs). The method takes advantage of flow injection MS methodology. It has been discovered according to the invention that non-covalent interactions can be detected using flow injection analysis to reduce the sample analysis time significantly. The survival of non-covalent interactions with the electrospray MS technique allows one to directly transfer intact compound-macromolecule complexes from a solution into a mass spectrometer without chromatographic separation of the mixture of the compound, macromolecule, and complex. Once the molecules are transferred to the mass spectrometer and ionized, they can be identified by their mass-to-charge ratio.

In general the prior art has used MS techniques to identify covalent binding interactions occurring between molecules. Prior to the instant invention several studies have suggested that electrospray ionization conditions may be mild enough that even relatively weak non-covalent interactions will survive the ionization process. This hypothesis, however, is controversial. Other researchers believe that it is not possible to detect non-covalent interactions using electrospray MS analysis because of the ionization conditions. It is thought that the detected complexes may be the result of a covalent interaction or may be non-specific.

The methods of the invention not only confirm the hypothesis that non-covalent interactions can be detected using electrospray ionization MS techniques but enable a novel method for the high throughput screening of a plurality of compounds for a non-covalent interaction with a target. Prior to the instant invention the ability to perform such a high throughput screening method was not known because of the conditions that researchers believed were required to maintain and detect a non-covalent interaction. The prior art methods which were used to examine the non-covalent binding interactions involved continuous infusion MS. As discussed in the background of the invention the use of an aqueous solution, which is an adequate environment for maintaining a non-covalent binding interaction, causes an unfavorable signal-to-noise ratio. In order to prevent the unfavorable signal-to-noise ratio, continuous infusion of the mixture is used so that the data may be acquired using the MCA mode of the mass spectrometer. The MCA mode which enhances a relatively weak signal, however, requires a sample analysis time on the order of greater than ten minutes. It was discovered according to the instant invention that non-covalent binding interactions can be screened using flow injection MS,

under conditions in which the sample analysis time is on the order of five minutes or even as low as one minute or 30 seconds, as compared to the sample analysis time of ten minutes for continuous infusion MS.

One problem associated with the continuous infusion mass spectrometry method of the prior art is that the dependence on manual manipulation of the solution in the syringe infusion pump and the relatively long acquisition time of the MCA mode allows for only few potential drug candidates to be screened against a given target in a reasonable amount of time. Moreover, it is completely impractical to use this approach to screen a target against the hundreds of thousands of compounds now being generated by combinatorial chemistry techniques. In addition, the relative binding strength of targets and compounds within different complexes would also be prohibitively labor-intensive and time-consuming to measure using a continuous flow method, where the analysis of the target-ligand solutions would be repeated several times under different potential energy conditions. The methods of the invention solve these problems arising from the prior art techniques.

The methods of the invention also solve the problem that continuous infusion electrospray MS is too labor-intensive and time-consuming for practical use in screening the hundreds of thousands of compounds that currently exist in pharmaceutical company collections, as well as the millions of compounds being developed using combinatorial chemistry techniques. Rather than using the continuous infusion method, researchers in the life sciences would continue to use the more conventional high-throughput screening methods that are primarily based on biological function because these methods are less expensive on a per unit basis than continuous infusion electrospray MS. The methods of the invention, however, provide a rapid high throughput method for screening hundreds of thousands of molecules in a relatively short time.

One aspect of the present invention is directed to a method for the rapid testing of a plurality of compounds for non-covalent interaction with a target. The method of the invention involves the steps of providing a continuous flow of a mobile phase into a mass spectrometer; providing settings for the mass spectrometer for detecting at least one target in the mobile phase; sequentially injecting individual samples of a plurality of mixtures, wherein each mixture comprises the at least one target and at least one compound of interest, into the mobile phase for delivery into the mass spectrometer; and obtaining a mass spectrum that indicates for each mixture the presence or absence of a complex of a target and a compound of interest. Preferably,

each sample is completely injected into the mobile phase within less than 5 minutes, and more preferably within less than 1 minute and even more preferably within less than 30 seconds. In another embodiment, each sample is analyzed within less than 5 minutes, and more preferably within less than 1 minute and even more preferably within less than 30 seconds.

Typically, the mass spectrometer is tuned to more accurately detect the target in the mobile phase. This is accomplished, in part, by adjusting the mobile phase to optimize overall mass spectrometric sensitivity while maintaining optimum solution equilibria for each mixture. The mass spectrometer may also be tuned by setting a minimum detection threshold based on the relative strength of interaction between the at least one target and the at least one compound of interest. This is preferably accomplished utilizing automated flow-injection analysis up-front collision induced dissociation mass spectrometry.

The method of the invention may be multiplexed for higher throughput by using mixtures that contain one target and a plurality of compounds of interest, one compound of interest and a plurality of targets, or a plurality of compounds of interest and a plurality of targets. In a preferred embodiment the target is a biological target. Preferably the biological target is a receptor protein.

Each of the mixtures that are tested using the method of the invention are preferably formed in a plurality of addressable locations, such as the individual wells in a multiple-well plate. The samples are then sequentially obtained from the addressable locations, and sequentially injected into the mobile phase for delivery to the mass spectrometer, such as by obtaining the samples from a multiple-well plate using an autosampler.

In one embodiment of the invention, at least one complex of a target and compound of interest are dissociated to determine the relative strength of the interaction between the target and the compound of interest within each complex. A preferred method for dissociating the complex is through the use of automated selected reaction monitoring flow-injection analysis mass spectrometry. The identities of one or more of the targets and the identities of one or more of the compounds of interest that complex with the targets may be determined based on the molecular mass of the dissociated targets and compounds of interest.

According to another aspect of the invention a method for rapid determination of relative non-covalent binding affinity of at least one compound of interest and at least one target is provided. The method includes the steps of providing a continuous flow of a mobile phase into a mass spectrometer; providing settings for the mass spectrometer for detecting at least one target

in the mobile phase; sequentially injecting individual samples of a plurality of mixtures, wherein each mixture comprises the at least one target and at least one compound of interest, into the mobile phase for delivery into the mass spectrometer; dissociating at least one complex of a target and compound of interest; and obtaining a mass spectrum that indicates for each mixture the relative strength of the interaction between the target and the compound of interest within each complex based on ability of the complex to be dissociated. Preferably, each sample is completely injected into the mobile phase within less than 5 minutes, and more preferably within less than 1 minute and even more preferably within less than 30 seconds. In another embodiment, each sample is analyzed within less than 5 minutes, and more preferably within less than 1 minute and even more preferably within less than 30 seconds.

In one embodiment the step of disassociating at least one complex is performed by applying a series of potential energies of increasing strength to the mixtures as each mixture is transferred from a solution phase to a gas phase in the spectrometer. Preferably the mass spectrum indicates for each mixture the potential energy that was sufficient to dissociate the complex, the potential energy being indicative of the non-covalent binding affinity of a complex between the target and the compound of interest.

In one embodiment the dissociation of the complex is conducted utilizing automated flow-injection analysis up-front collision induced dissociation mass spectrometry, and applying different up-front cone voltages to create different potential energies. In another embodiment the dissociation of the complex is conducted utilizing automated selected reaction monitoring flow-injection analysis mass spectrometry.

According to another embodiment of the invention the plurality of mixtures contains a single compound of interest and a single target and a complex thereof and wherein a series of potential energies of increasing strength is applied to the mixtures as each mixture is transferred from a solution phase to a gas phase, and obtaining a mass spectrum that indicates for each mixture which potential energy was sufficient to dissociate the complex, the potential energy being indicative of the non-covalent binding affinity of a complex between the target and the compound of interest.

In yet another embodiment the plurality of mixtures each contain a single target and wherein the plurality of mixtures each contain a different compound of interest. In a different embodiment the plurality of mixtures each contain a single compound of interest and wherein the plurality of mixtures each contain a different target.

The method of the invention in another embodiment includes the step of forming the plurality of mixtures in a plurality of addressable locations. Prefereably each addressable location is an individual well in a multiple-well plate. The method may also include the steps of sequentially obtaining samples from the addressable locations, and sequentially injecting the samples into the mobile phase for delivery to the mass spectrometer. Preferably the samples are obtained from a multiple-well plate using an autosampler.

In another aspect the invention is a method for rapid analysis of structural binding properties of a compound that forms a non-covalent interaction with a target. The method includes the steps of generating a database of relative binding affinities by performing the method described above for determining relative non-covalent binding affinity and comparing the structures of the compounds of interest having the highest relative binding affinities to determine structural similarities amongst the compounds of interest having the highest relative binding affinities, wherein the structural similarities are indicative of structural binding properties of a compound that forms a non-covalent interaction with the target.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each method.

Brief Description of the Drawings

Fig. 1 is a chemical structure of insulin ligands.

Fig. 2 is a mass spectra of insulin and insulin-ligand complexes.

Fig. 3 is a summary of mass spectrometry results.

Fig. 4 is a summary of mass spectrometry results.

Fig. 5 is a graph depicting relative binding strength of 9 insulin-ligand complexes.

Fig. 6 is a FIA chromatogram of FKBP/Rapamycin solution using a Micromass LCT.

Fig. 7 is a mass spectra of FKBP/Rapamycin solution using a Micromass LCT.

Fig. 8 is a mass spectra of Avidin/Biotin solution using a Micromass LCT.

Detailed Description

The present invention relates to an automated, high throughput method for screening a plurality of chemical compounds to identify interactions with one or more macromolecular targets. The method allows one of ordinary skill in the art to rapidly detect interactions between

a given biological target and a compound of interest, and also provides a method to determine the relative strength of the interaction between the target and compound.

An electrospray MS technique of detecting non-covalent complexes of a macromolecular target and a chemical compound can be applied to the screening of targets with chemical compounds for the discovery of new drugs. Drug molecules often form weak, non-covalent bonds with specific proteins or other biological molecules in a manner that is analogous to a lock and key, and thereby assert their influence on the body through these non-covalent bonds. The better a drug molecule can bind to a target, the stronger the efficacy (and often specificity) the drug will exhibit.

As used herein, the term "plurality of compounds of interest" refers to two or more compounds, and includes but it is not limited to, an array or library of compounds generated using combinatorial chemistry techniques, and also includes, but is not limited to, libraries of natural products, libraries of biological molecules (e.g., polypeptides, polynucleotides, oligosaccharides), libraries of compounds produced with combinatorial biology techniques, and any other collection of chemical compounds.

A "target" as used herein is any structure which can form a non-covalent binding interaction with a compound such as a ligand. Preferably the target is a biological target. The preferred embodiments of the invention are described in terms of a biological target, but the description is not intended to limit the scope of the invention to biological targets. It is apparent to one of ordinary skill in the art that the appended claims cover all targets. A target can include multimeric structures as well as a monomeric structure.

A "biological target" as used herein is a target which is ordinarily found in a living system or which interacts with a compound such as a ligand which is ordinarily found in a living system. Biological targets can be natural compounds or synthetic compounds and include for example but are not limited to receptors, polypeptides, proteins, polynucleotides, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), carbohydrates, polysaccharides, and lipids.

Automated electrospray MS using flow injection analysis (FIA) has been applied according to the invention to identify non-covalent complexes. Instead of using a syringe infusion pump to deliver analytes, the method of the invention uses an automated sampling device, such as an HPLC pump equipped with an autosampler, to transport a specific amount of sample in a continuous stream of solvent (mobile phase) to the mass spectrometer via a loop

injection. With the method of the invention, a continuous stream of mobile phase is established from the HPLC system to the mass spectrometer, in order to transport the aliquot of sample. Because the mobile phase is only the conduit to transport the aliquot of sample to the mass spectrometer, its composition can be adjusted to optimize the overall mass spectrometric sensitivity while maintaining optimum solution equilibria established for each target-ligand mixture. The mobile phase velocity and the amount of tubing connecting the HPLC to the MS determines the time that the injected aliquot will reach the mass spectrometer. By increasing the mobile phase flow, the injected sample will reach the mass spectrometer faster, increasing the overall throughput. However, the faster the sample moves throughout the system, the smaller number of discrete scans that can be obtained. The best instrumental parameters can be identified by those of ordinary skill in the art in order to maximize both sensitivity and throughput. Although the overall cycle time from sample to sample will depend on the overall sensitivity of the instrument for the particular molecular target, the injection of the sample in the mobile phase is typically less than 1 minute, compared to about 10 minutes or more per samples for the continuous infusion process.

The MS can be "tuned", such that the macromolecular target or targets can be readily detected. This is accomplished using the infusion pump method which is well known in the art. Once the MS is tuned to detect the target, the MS will also be able to detect the compound of interest and any complexes that are formed by the macromolecular target and a compound of interest. An HPLC/MS interface is set up by connecting the HPLC to an electrospray probe, and the flow rate is optimized for maximum sensitivity for the target macromolecular compound. Typically, a flow rate of about 0.01 to about 0.2 ml/min, preferably about 0.05 to about 0.15 ml/min, is selected as the optimal flow rate. Such a flow rate provides sufficient sensitivity to obtain mass spectra of macromolecules, such as receptor proteins or receptor-ligand complexes, within about 5 minutes (total cycle from sample to sample injection), preferably about 2.5 minutes per sample. In addition, the mass spectrometric parameters can be optimized to eliminate fragmentation of any complexes formed, due to the application of cone voltages, source temperature, etc. Once a continuous flow of a mobile phase into the MS is established, several injections of the macromolecular solution are made in order to confirm that the FIA conditions are appropriate and optimized. When the appropriate conditions are maintained for the macromolecular target, several ligand solutions of interest are also injected using the established parameters in order to confirm that the instrumental parameters optimized for the

macromolecular target are indeed appropriate for detection of the small molecular weight ligands. When the instrumental parameters are optimized for detection of both the macromolecular target and the small molecular weight molecules, samples of the mixtures to be screened are injected into the mobile phase for analysis.

Typically, reaction mixtures of a macromolecular target, such as a biological receptor protein, and at least one compound of interest, such as potential drugs, are mixed, and placed individually in a 96-well microtitre plate, and sample mixtures are injected automatically from each well in the plate.

In a typical screening, an HPLC system is set up to provide a flow rate of less than about 0.2 ml/minute, preferably less than about 0.1 ml/minute, in order to provide an overall analysis cycle time from sample to sample in less than about 5 minutes, preferably less than about 1 minute or 30 seconds, without manual intervention. This is a great improvement over the methods of the prior art, which require more than 10 to 15 minutes to analyze a single sample with manual intervention by the operator for each sample.

Assuming each sample contains only molecules of a single compound of interest and a single target, plus solvent, the MS will produce, at most, 3 peaks or sets of peaks, depending on the amount of charge distribution that the macromolecule can stabilize. The peaks observed include a peak for the macromolecular target, a peak for the small molecular weight compound of interest in the sample, and, if the compound of interest has an interaction with the target, a peak or sets of peaks attributed to the non-covalent complex formed by the two molecules. The size of the peak for the complex indicates the relative strength of the interaction between the chemical compound and the macromolecular target. If the interaction is strong, a large peak will result; if the interaction is weak, a smaller peak will result.

The automated MS method of the invention is particularly useful for enabling high-throughput screening of compounds in combinatorial chemistry libraries to detect interaction between the compounds and a particular protein target by the rapid and direct detection of non-covalent receptor-ligand complexes in the gas phase in a manner that approximates solution phase equilibria.

Another aspect of the invention provides a method for determining the relative strength of the interaction between a biological target and various compounds of interest by comparing the binding strength of each ligand in relation to the other members of a particular set using automated FIA up-front collision induced dissociation (CID) MS. In this method, different up-

front cone voltages are applied so that different potential energies are created. The greater the potential energy in the instrument, the more energy imparted to the complex being transferred from the solution phase into the gas phase. If the imparted energy is greater than the binding energy involved in the non-covalent interaction, the complex will dissociate prior to entering the first MS and the complex will not be detected. Thus, by changing the cone voltage, only complexes of a certain binding energy will be seen. This allows one to easily set a minimum interaction strength threshold that allows one to increase or decrease the number of reported interactions (commonly referred to as "hits"). The greater the strength of an interaction, the more likely the interaction reflects a specific molecular recognition.

The results obtained from FIA MS and up-front CID MS can be used to screen large number of compounds (in the hundreds of thousands or more) for interaction with specific biological targets. These compounds can be further studied by an automated selected reaction monitoring (SRM) FIA MS method. Samples are introduced into the mass spectrometer using FIA and maintaining the cone voltage constant. The first MS analyzer (MS1) is set to pass only masses equal to that of the complex. The selected parent ion, such as a receptor-ligand complex, is directed into a collision cell, where it is fragmented by collision induced dissociation (CID) by introducing argon, helium or other neutral gases. The collision energy applied to the selected parent dissociates the complex into a receptor and a ligand which are then separated and detected by the second mass analyzer (MS2). This FIA-SRM-MS technique is monitored as a function of increasing collision energy under a constant cone voltage and collision gas thickness, allowing rapid monitoring of the relative binding strengths by dissociating the complex in the gas phase. Because only one ion is selected by MS1 at any given time for CID, this approach provides increased selectivity and specificity to monitor the relative strength of binding interactions.

In addition, it is also possible to multiplex the screening process with the method of the invention. Multiplexing is performed by mixing one compound of interest with a plurality of targets, by mixing a plurality of compounds of interest with one target, or by mixing a plurality of targets with a plurality of compounds of interest. The composition of any complex can be determined from the molecular mass of the complex, i.e., the molecular mass of the complex is the sum of the compound and the target that comprise the complex. In a typical random screening against a single target, only one compound in 10,000 will interact with a target and thereby form a complex with the target. Therefore, when the method of the invention is multiplexed, the odds for the formation of multiple complexes in a single sample are very low.

However, in the event that two or more different complexes are formed, the multiple MS peaks observed can be differentiated and identified based on their different molecular masses. Of course, this approach will not address the rare situation where multiple complexes with the same molecular mass are observed. This rare situation may be eliminated with careful selection of targets and compounds in a multiplexed screen to ensure that either the targets or compounds have distinct molecular masses such that isobaric complexes of different molecular structures will not be observed. Alternatively, the few isobaric complexes that are likely to occur may be re-analyzed with LC, LC/MS, or MS/MS to identify the components.

The method of the invention allows one of ordinary skill in the art to rapidly detect interactions between a given biological target and a compound of interest, and also provides a method to determine the relative strength of the interaction between the target and compound. Once the compounds of interest which bind to the target compound have been identified the biological function of the compound of interest on the target can be assessed. For instance, if the target compound is a receptor, a biological assay can be performed to determine whether the binding compound functions as an agonist or an antagonist. An assay based on biological function can be performed to confirm that the compound has a desired activity. The method of the invention dramatically reduces the number of compounds that require biological testing, such that even the most expensive *in vivo* animal tests become practical in many cases, based on the assumption that only about one compound in 10,000 will exhibit sufficiently strong interaction with a biological target to warrant confirmatory biological testing.

The invention therefore provides a rapid, cost-effective method to screen large numbers of compounds to detect a specific interaction with a biological target without knowledge of the biological function of the target or knowledge of the biological system with which that target interacts. The method avoids the need for biological assay development and validation in a high-throughput format, although some assay development is useful unless whole animal testing is used. The expense of biological screening methods is reduced by decreasing the numbers of compounds that must be tested for a particular target. The per unit cost of screening by the method of the invention is less expensive than most screens based on biological function.

Although the present invention was developed during an investigation for an affinity ligand for a biologically important protein, to be used as a stationary phase for affinity chromatography, it will be readily understood by one of ordinary skill in the art that the method of the invention is applicable wherever any form of molecular recognition event occurs, and is

useful in determining lead compounds for many applications. In affinity chromatography, a mixture of molecules is passed through an affinity column that contains immobilized ligands with an affinity for one of the molecules in the mixture. That molecule will be retained in the column as the other components in the mixture pass through the column. A solvent that causes the molecule to be released from the ligands is then passed through the column, and a solution of the substantially pure molecule is obtained. With the method of the invention, large numbers of potential affinity ligands may be screened in a short period of time.

In contrast to the continuous infusion electrospray MS method, the present invention provides an automated, high throughput MS and MS/MS method for rapid sequential screening of a plurality of compounds of interest to determine which form non-covalent complexes with macromolecular targets. Typically, the analysis cycle time from sample to sample, each containing a mixture of at least one compound of interest and at least one macromolecular target compound takes less than about 5 minutes and, preferably, less than about 1 minute and more preferably less than about 30 seconds.

The invention also encompasses a method for analyzing the structural binding properties of a compound. The method can be accomplished by generating a database of relative binding affinities of a plurality of similar compounds with a target. The compounds which have the highest binding affinities for the target reveal the structural binding properties of the compound.

The only limitation of the methods described herein is based on the state of the art of the equipment. Using the MS equipment available today it may be difficult to detect large, high molecular mass targets. As will be readily understood by one of ordinary skill in the art, as mass spectrometers are developed that allow the detection of molecules of higher molecular mass, the method of the invention can be extended to larger targets.

EXAMPLES

The following non-limiting examples are merely illustrative of the preferred embodiments of the present invention, and are not to be construed as limiting the invention, the scope of which is defined by the appended claims.

Example 1

Materials

Human recombinant (hr) insulin purchased from Sigma (St. Louis, MO) was used as a

target protein receptor. An amino acid arylidene diamine (AA-ADA) combinatorial library of insulin potential ligands were synthesized by parallel synthesis. Examples of parallel synthesis mixtures and parallel synthesis methods are provided in U.S.S.N. 08/177,497, filed January 5, 1994 and its corresponding PCT published patent application W095/18972, published July 13, 1995 and U.S. Patent No. 5,712,171 granted January 27, 1998 and its corresponding PCT-published patent application W096/22529, which are hereby incorporated by reference. Eighty potential insulin ligands synthesized and disposed in individual wells of a 96-well microtitre plate were tested for screening insulin-ligand complexes. Nine purified AA-ADA/dimer insulin ligands (AQ41438 - AQ41447, Figure 1) were studied for the binding strength with the receptor by the up-front CID MS and MS/MS.

Sample Preparation

A stock solution of 100 μM hr insulin was prepared in 3% acetic acid. A working solution of 25 μM hr insulin was freshly prepared before use. Eighty AA-ADA compounds, individually synthesized and disposed in individual wells of a 96-well microtitre plate, were originally dissolved in 100% DMSO to give a concentration of 5 mM of each. These compounds were respectively diluted with 100% acetonitrile (1 to 5 dilution) to obtain a concentration of 1 mM. Then 900 μl of hr insulin (25 μM) and 100 μl of each AA-ADA compound (1 mM) were mixed for a total mixture volume of 1 ml, and an approximate molar ratio of 1 to 5 (hr insulin to AA-ADA/dimer) in each well.

L-Lysine-ADA/dimer (AQ41440/dimer), known to bind with hr insulin, and L-lysine-ADA/monomer (AQ41440/monomer), known to disassociate from hr insulin, were synthesized and purified for use as controls. Eight QC samples were placed and analyzed in a 96-well microtitre plate between every other 10 samples. These QC samples included hr insulin, AQ41440/monomer, AQ41440/dimer, a mixture of insulin + AQ41440/monomer, and a mixture of insulin + A141440/dimer. 10 μl of each sample was injected through a loop injection into a mass spectrometer.

Nine targeted insulin ligands (AQ41438 - AQ41445, and AQ41447) were individually dissolved in 100% ACN to give a concentration of 1 mg/ml. Then 900 μl of insulin (25 μM) was mixed with 100 μl of each ligand prior to MS analysis.

Instrumentation

A Hewlett Packard 1050 pump equipped with a Hewlett Packard 1050 diode array detector was used to deliver solvent (50/50 acetonitrile/water in 0.1% formic acid) at a flow rate of 0.1 ml/minute. Samples (10 µl per injection) were injected through an Hitachi AS-400 intelligent autosampler to a mass spectrometer. A Micromass Quattro triple quadrupole tandem mass spectrometer was operated in the positive ion mode using an electrospray LC/MS interface. Mass spectrometric conditions were optimized using a corona voltage of 3 kV, source temperature of 30°C, and scanning time of 3 seconds. Various cone voltages from 15V to 40V were used to further screen receptor-ligand complexes. Data were acquired under MS1 full scan from *m/z* 350 to *m/z* 1500 for 2.5 minutes. Liquid nitrogen from a dewar was used as both a bath gas and a nebulizer gas. Argon was used as collision gas for MS/MS experiments. Collision energies were applied between 0 and 200 eV to study the binding strength of receptor-ligand complexes. Automatic flow-injection analysis (FIA) MS was performed to screen these potential insulin targeted compounds.

Results

A mass spectrum of free hr insulin (25 µM in 3% acetic acid) was obtained in Figure 2C. Under acidic conditions, multiply protonated charged ions from $(M + 4H)^{4+}$ to $(M + 6H)^{6+}$ were observed at m/z 1451.8, 1162.0, 968.9 to give hr insulin a calculated molecular weight of 5807 Daltons in agreement with its theoretic molecular weight of 5807.6 Daltons. The mass-to-charge regions between the three charge states are of interest for detecting complexes. Insulin was mixed with approximate 5-fold excess of each potential ligand and the mixture was automatically analyzed by FIA MS. A new signal appeared at m/z 1317 corresponding to the insulin-AQ41440/dimer complex in the 5+ charge state (Figure 2A); however, there was no signal shown at m/z 1254 for the insulin-AQ41440/monomer complex in the 5+ charge state (Figure 2B) in agreement with biological results. Hence, AQ41440/monomer and AQ41440/dimer were chosen as controls to confirm specific insulin-ligand complexes for screening a combinatorial library of potential insulin ligands.

Although it was believed that the use of 50/50 ACN/H_2O in 0.1% formic acid as a mobile phase to detect protein-ligand complexes could denature proteins and affect their binding activity, it has been recently reported that the structure conformation of insulin was stabilized by acid. In addition, insulin specifically tends to be aggregated to a hexamer in the presence of divalent metal ions. Mixtures of water and acids or water and organic solvents were used to

reduce the tendency of insulin to aggregate. Therefore, the presence of 0.1% formic acid and acetonitrile in mobile phase can not only prevent insulin from aggregation, but also offers a good environment for performing a mass spectrometer.

An eighty AA-ADA combinatorial library of hr insulin potential ligands, synthesized and disposed in individual wells of a 96-well microtitre plate, was first examined by automatic FIA MS to confirm the synthesis of these compounds. Each flow injection analysis took only 1.5 minutes. Using the OpenLynx diversity software provided by Micromass, Inc., instance MS summary results (Figures 3 and 4) were reported for each compound in each well. Figure 3A showed that all eighty AA-ADA/monomers were made. Nevertheless, 55 out of 80 AA-ADA/dimers were synthesized for the array, indicating that these individual 55 compounds contained both AA-ADA/monomer and AA-ADA/dimer, and the rest 25 compounds contained only AA-ADA/monomers. After insulin was added, neither one of insulin-AA-ADA/monomer complex was observed by MS (Figure 3B), whereas 28 out of 55 AA-ADA/dimers were found to be bound with insulin at a cone voltage of 15 V (Figure 4A). These results indicate that there were specific insulin-ligand binding complexes detected in the gas phase by MS. In fact, these non-covalent interactions between the receptor and ligands might occur at a non-specific binding site in solutions. Therefore, different potential energies (cone voltages) were applied to investigate these non-covalent complexes. From cone voltages of 20 V to 25 V (Figure 4B and 4C), only 8 to 10 complexes were detected, in comparison with 28 complexes found at the cone voltage of 15 V. It is believed that weaker associations, possibly resulting from non-specific interactions, might dissociate from the application of gentle potential energies that are high enough to knock out those non-specific interactions, but not to dissociate the specific receptor-ligand complexes from the specific binding pocket. Whenever a higher potential energy is used, only stronger non-covalent binding complexes in the gas phase survive. Figure 4D shows that there were only two insulin-ligand complexes in wells E4 and F4 observed at a cone voltage of 30 V. In addition, Figure 4A-4D show that these insulin-ligand complexes were found mostly in row 4 and column E, implying that these ligands made from particular chemical components X and Y used in row 4 and column E tended to associate with insulin.

Nine purified AA-ADA/dimer compounds (AQ41438 - AQ41445, AQ41447; Figure 1), showing binding activity with insulin from biological assays, were chosen for further studying the binding association between insulin and ligands by up-front CID MS using various cone voltages. Both 5+ and 5+ charged states of insulin-AA-ADA/dimer complexes were detected

using this automatic flow injection MS analysis. Since the complex ion current can vary from injection to injection, the ion current ratio of the complex to the free insulin, used as an internal standard, at 5+ charged state was monitored as a function of increasing up-front CID potential energy. At lower cone voltages (10 - 15 V), six ligands (AQ41439, AQ41440, AQ41441, AQ41442, AQ41444, AQ41445) showed about 20 to 40% relative binding with insulin. Two ligands (AQ41438 and AQ41447) showed 60% relative binding, and one ligand (AQ41443) showed only 10% relative binding (Figure 5). Results shown in Figure 5 suggested that AQ41438 and AQ41447 might not only associate at a non-specific binding site with insulin, but may also interact with the receptor in the specific binding pocket at a low cone voltage (10 V). Undoubtedly AQ41443 showed the least some binding (10%) with insulin, implying that this is the weakest ligand compared to other 8 ligands. It was interesting to note that AQ41444 (ethyl/D-ornithine-ADA) are isomers. However, AQ41444 would bind with insulin at least twice as strongly as AQ41443 (Figure 5), confirming that detected complex ions in the gas phase would agree with the specific binding complexes observed in the solution. Nevertheless, this flow injection electrospray MS technique demonstrates a much faster and more direct analysis to study receptor-ligand complexes.

Example 2:

A second example of non-covalent interaction that was studied was the well known complex of FKBP (MW 11,822 Daltons) with Rapamycin. An 8.5 micromolar solution of FKBP was prepared in 10 millimolar ammonium acetate (pH~7). In addition, a 109 micromolar solution of Rapamycin was prepared in methanol. Nine parts of the FKBP were combined with 1 part of the Rapamycin solution to prepare a 7.7:1 micromolar ratio, respectively. Two microliters of the resulting solution were injected into a Micromass electrospray time-of-flight instrument equipped with a Shimadzu SCL 10 Avp HPLC system pumping 10 millimolar ammonium acetate (pH~7) at 0.1 mL/min. The source temperature of the mass spectrometer was 30C, the cone voltage was 40V and the mass range of 200-3000 amu was scanned in 0.1 seconds. The FIA chromatogram obtained from this analysis is given in Figure 6. Note that because of the faster scan rate possible with a time-of-flight instrument, there are more scans per unit time available, and therefore an enhancement in total sensitivity. The mass spectrum of the flow injection analysis of FKBP with Rapamycin is shown in Figure 7. The quality of the given spectrum, obtained by FIA/MS, is better than typically obtained by continuous infusion

compared to quadrupole instruments. Note that the free protein, the free Rapamycin and the protein/Rapamycin complex are easily distinguished in the spectrum with excellent signal-to-noise ratio.

This method was further investigated using another well-known non-covalent complex that includes a higher molecular weight protein, avidin (MW 63,872 Daltons) complexing with four biotin molecules. A 15.6 micromolar solution of avidin was prepared in 10 millimolar ammonium acetate (pH~7). A 4.1 millimolar solution of biotin was also prepared in 10 millimolar ammonium acetate (pH~7). Nine parts of the avidin were combined with 1 part of the biotin solution to prepare a 14:410 micromolar ratio, respectively. A 10 microliter aliquot of the avidin/biotin solution was injected into the electrospray time-of-flight instrument system identified above. The only difference in the operating parameters were as follows: cone voltage was 60-V and mass range was 200-10,000 in 0.1 seconds. The mass spectrum obtained from the 10 microliter flow injection analysis is given in Figure 8 and compared to the flow injection analysis spectrum obtained from the avidin solution alone. the mass shift indicated for the avidin/biotin spectrum corresponds to the addition of the biotin molecules. Because of the relatively large nature of the protein, as well the heterogeneous nature of avidin due to a number of glycosylations, and the possibility of binding from one to four biotin molecules on each avidin, the resolution of the peaks are compromised and it is difficult to assign an accurate mass for each charge state. However, the avidin/biotin system represents one of the more difficult challenges and the fact that we are able to obtain the data shown in Figure 8 indicates the power of this technique.

Each of the foregoing patents, patent applications and references that are recited in this application are herein incorporated in their entirety by reference. Having described the presently preferred embodiments, and in accordance with the present invention, it is believed that other modifications, variations and changes will be suggested to those skilled in the art in view of the teachings set forth herein. It is, therefore, to be understood that all such variations, modifications, and changes are believed to fall within the scope of the present invention as defined by the appended claims.

What is claimed is:

CLAIMS

1. A method for rapid testing of a plurality of compounds of interest for non-covalent interaction with at least one target, comprising:

providing a continuous flow of a mobile phase into a mass spectrometer;

providing settings for the mass spectrometer for detecting at least one target in the mobile phase;

sequentially injecting individual samples of a plurality of mixtures, wherein each mixture comprises the at least one target and at least one compound of interest, into the mobile phase for delivery into the mass spectrometer; and

obtaining a mass spectrum that indicates for each mixture the presence or absence of a complex of at least one target and a compound of interest.

2. The method of claim 1, wherein the mixture contains one target and a plurality of compounds of interest.

3. The method of claim 1, wherein the mixture contains one compound of interest and a plurality of targets.

4. The method of claim 1, wherein the mixture contains a plurality of compounds of interest and a plurality of targets.

5. The method of claim 1, wherein the target is a biological target.

6. The method of claim 5, wherein the biological target is a receptor protein.

7. The method of claim 1, which further comprises adjusting the mobile phase to optimize overall mass spectrometric sensitivity while maintaining optimum solution equilibria for each mixture.

8. The method of claim 1, which further comprises tuning the mass spectrometer to more accurately detect the target in the mobile phase.

9. The method of claim 8, which further comprises setting a minimum detection threshold based on the relative strength of interaction between the at least one target and the at least one compound of interest.

10. The method of claim 9, wherein the minimum detection threshold is set utilizing automated flow-injection analysis up-front collision induced dissociation mass spectrometry.

11. The method of claim 8, which further comprises forming the plurality of mixtures in a plurality of addressable locations.

12. The method of claim 11, wherein each addressable location is an individual well in a multiple-well plate.

13. The method of claim 11, which further comprises sequentially obtaining samples from the addressable locations, and sequentially injecting the samples into the mobile phase for delivery to the mass spectrometer.

14. The method of claim 13, which further comprises obtaining the samples from a multiple-well plate using an autosampler.

15. The method of claim 1, which further comprises dissociating at least one complex of at least one target and compound of interest to determine the relative strength of the interaction between the target and the compound of interest within each complex.

16. The method of claim 15, wherein the dissociation of the complex is conducted utilizing automated selected reaction monitoring flow-injection analysis mass spectrometry.

17. The method of claim 16, wherein the identities of one or more targets and the identities of one or more compounds of interest that complex with the targets are determined based on the molecular masses of the dissociated targets and compounds of interest.

18. The method of claim 1 wherein each sample is completely injected into the mobile phase

within less than 5 minutes.

19. The method of claim 1 wherein each sample is completely injected into the mobile phase within less than 1 minute.

20. The method of claim 1 wherein each sample is completely injected into the mobile phase within less than 30 seconds.

21. The method of claim 1 wherein each sample is analyzed within less than 5 minutes.

22. The method of claim 1 wherein each sample is analyzed within less than 1 minute.

23. The method of claim 1 wherein each sample is analyzed within less than 30 seconds.

24. A method for rapid determination of relative non-covalent binding affinity of at least one compound of interest and at least one target, comprising:

providing a continuous flow of a mobile phase into a mass spectrometer;

providing settings for the mass spectrometer for detecting at least one target in the mobile phase;

sequentially injecting individual samples of a plurality of mixtures, wherein each mixture comprises the at least one target and at least one compound of interest, into the mobile phase for delivery into the mass spectrometer;

dissociating at least one complex of at least one target and compound of interest; and

obtaining a mass spectrum that indicates for each mixture the relative strength of the interaction between the target and the compound of interest within each complex based on ability of the complex to be dissociated.

25. The method of claim 24, wherein the step of disassociating at least one complex is performed by applying a series of potential energies of increasing strength to the mixtures as each mixture is transferred from a solution phase to a gas phase in the spectrometer.

26. The method of claim 25, wherein the mass spectrum indicates for each mixture the

potential energy that was sufficient to dissociate the complex, the potential energy being indicative of the non-covalent binding affinity of a complex between the target and the compound of interest.

27. The method of claim 24, wherein the dissociation of the complex is conducted utilizing automated flow-injection analysis up-front collision induced dissociation mass spectrometry, and applying different up-front cone voltages to create different potential energies.

28. The method of claim 24, wherein the dissociation of the complex is conducted utilizing automated selected reaction monitoring flow-injection analysis mass spectrometry.

29. The method of claim 24, wherein the plurality of mixtures contains a single compound of interest and a single target and a complex thereof and wherein a series of potential energies of increasing strength is applied to the mixtures as each mixture is transferred from a solution phase to a gas phase, and obtaining a mass spectrum that indicates for each mixture which potential energy was sufficient to dissociate the complex, the potential energy being indicative of the non-covalent binding affinity of a complex between the target and the compound of interest.

30. The method of claim 24, wherein the plurality of mixtures each contain a single target and wherein the plurality of mixtures each contain a different compound of interest.

31. The method of claim 24, wherein the plurality of mixtures each contain a single compound of interest and wherein the plurality of mixtures each contain a different target.

32. The method of claim 24, further comprising forming the plurality of mixtures in a plurality of addressable locations.

33. The method of claim 32, wherein each addressable location is an individual well in a multiple-well plate.

34. The method of claim 32, which further comprises sequentially obtaining samples from the addressable locations, and sequentially injecting the samples into the mobile phase for

delivery to the mass spectrometer.

35. The method of claim 34, which further comprises obtaining the samples from a multiple-well plate using an autosampler.

36. The method of claim 24, wherein each sample is completely injected into the mobile phase within less than 1 minute.

37. The method of claim 24, wherein each sample is completely injected into the mobile phase within less than 30 seconds.

38. The method of claim 24, wherein each sample is analyzed within less than 1 minute.

39. The method of claim 24, wherein each sample is analyzed within less than 30 seconds.

40. A method for rapid analysis of structural binding properties of a compound that forms a non-covalent interaction with at least one target, comprising:

generating a database of relative binding affinities by performing the method of claim 24; and

comparing the structures of the compounds of interest having the highest relative binding affinities to determine structural similarities amongst the compounds of interest having the highest relative binding affinities, wherein the structural similarities are indicative of structural binding properties of a compound that forms a non-covalent interaction with the target.

CHEMICAL STRUCTURES OF INSULIN LIGANDS

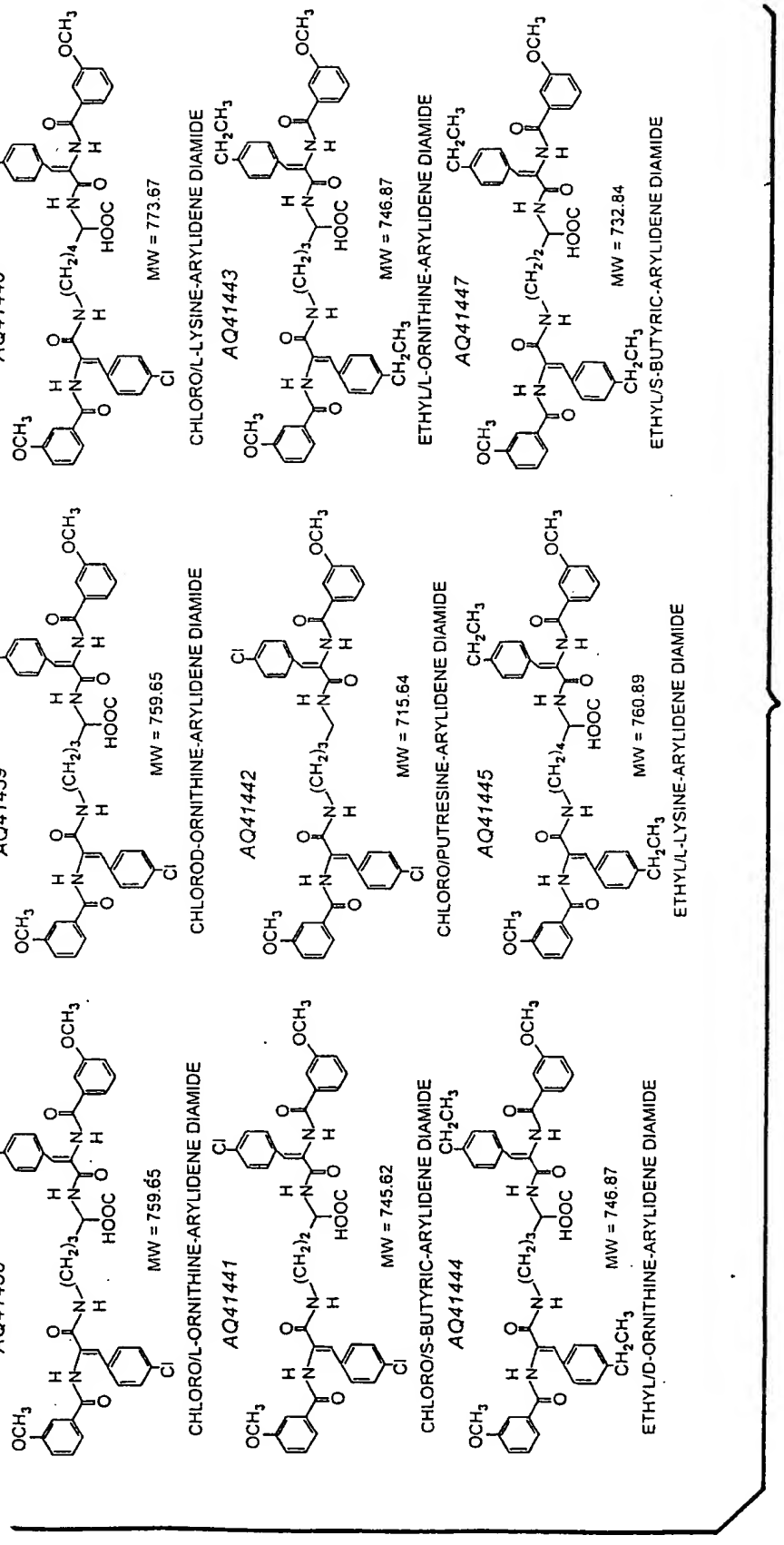


Fig. 1

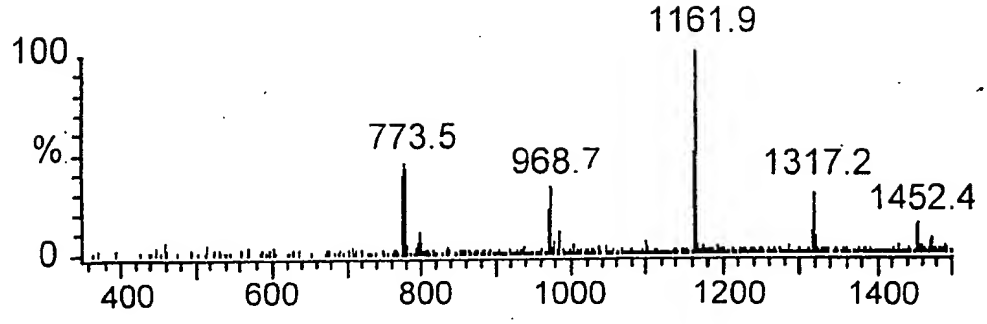


Fig. 2A

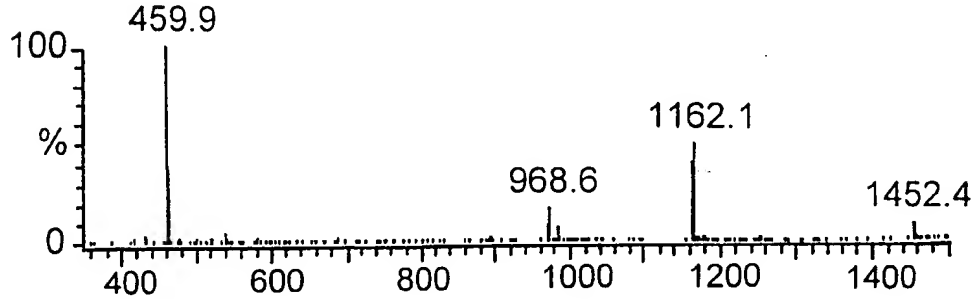


Fig. 2B

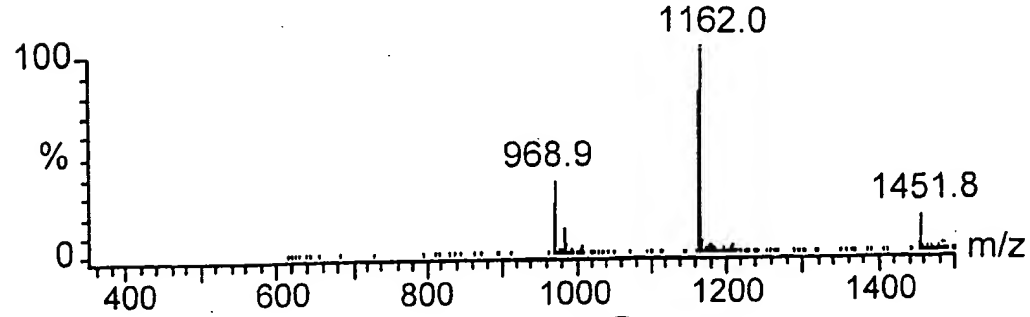


Fig. 2C

Fig. 2

Fig. 3

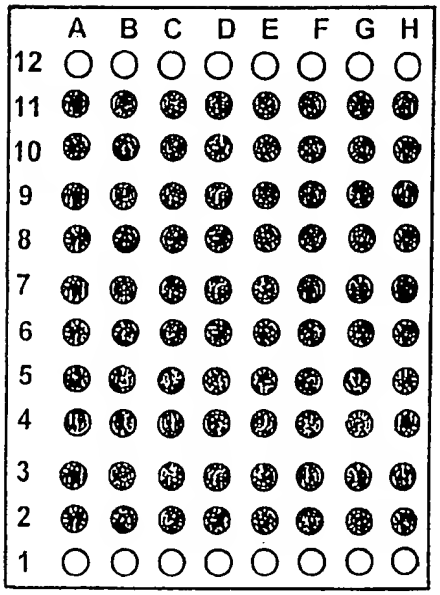


Fig. 3A

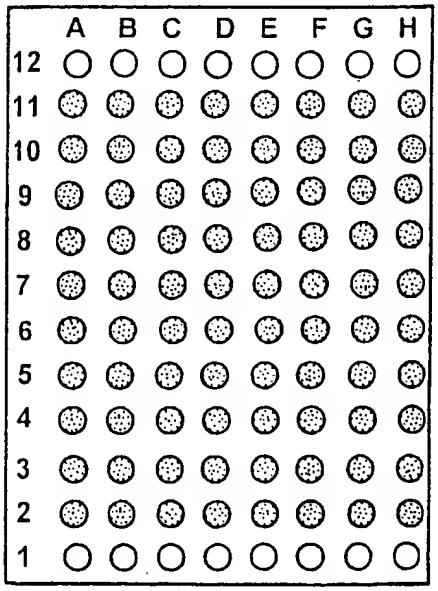


Fig. 3B

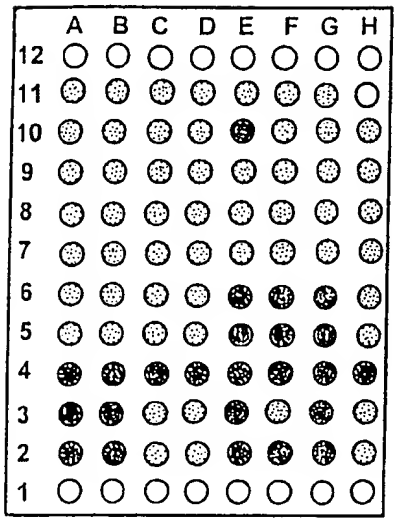


Fig. 4A

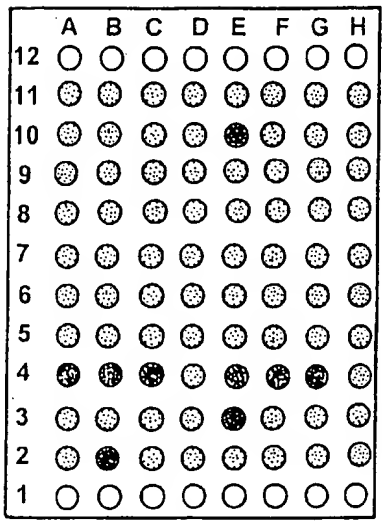


Fig. 4B

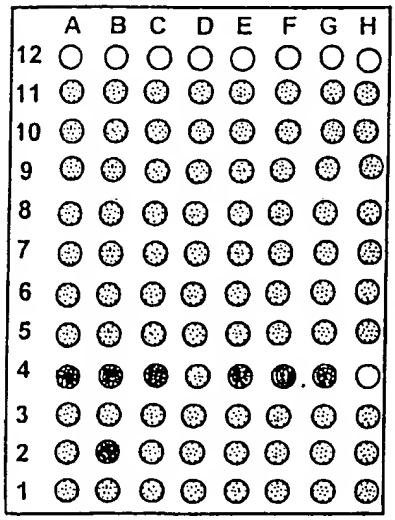


Fig. 4C

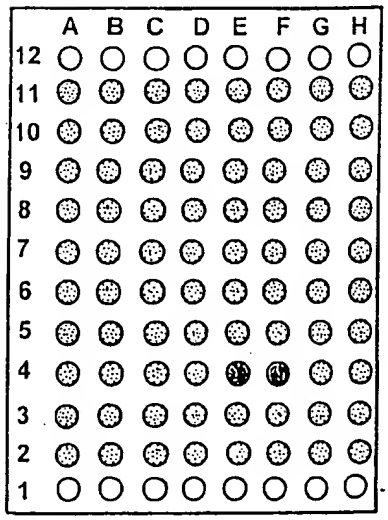
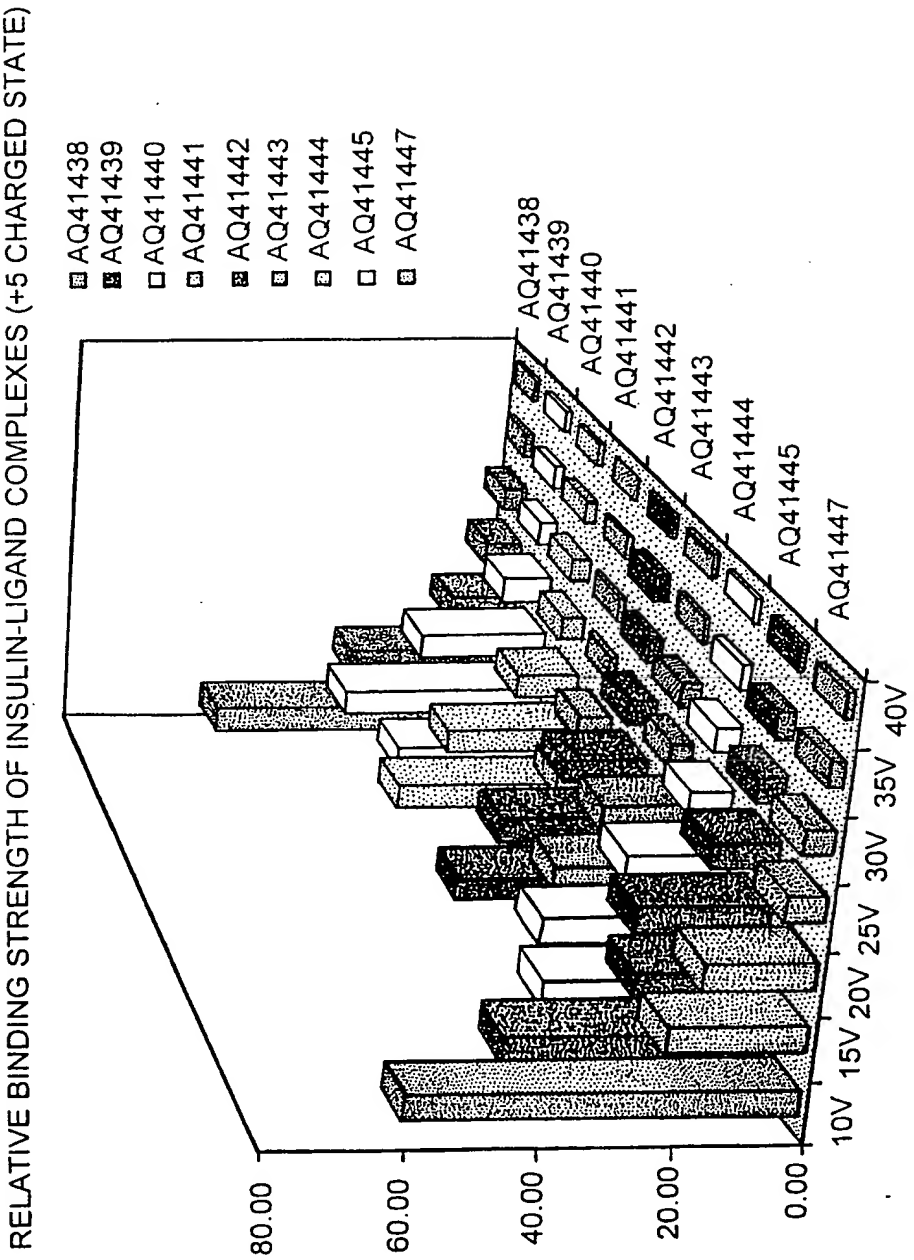


Fig. 4D

Fig. 4


RELATIVE BINDING STRENGTH OF INSULIN-LIGAND COMPLEXES (+5 CHARGED STATE)
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AQ41439
AQ41440
AQ41441
AQ41442
AQ41443
AQ41444
AQ41445
AQ41447
80.00
60.00
40.00
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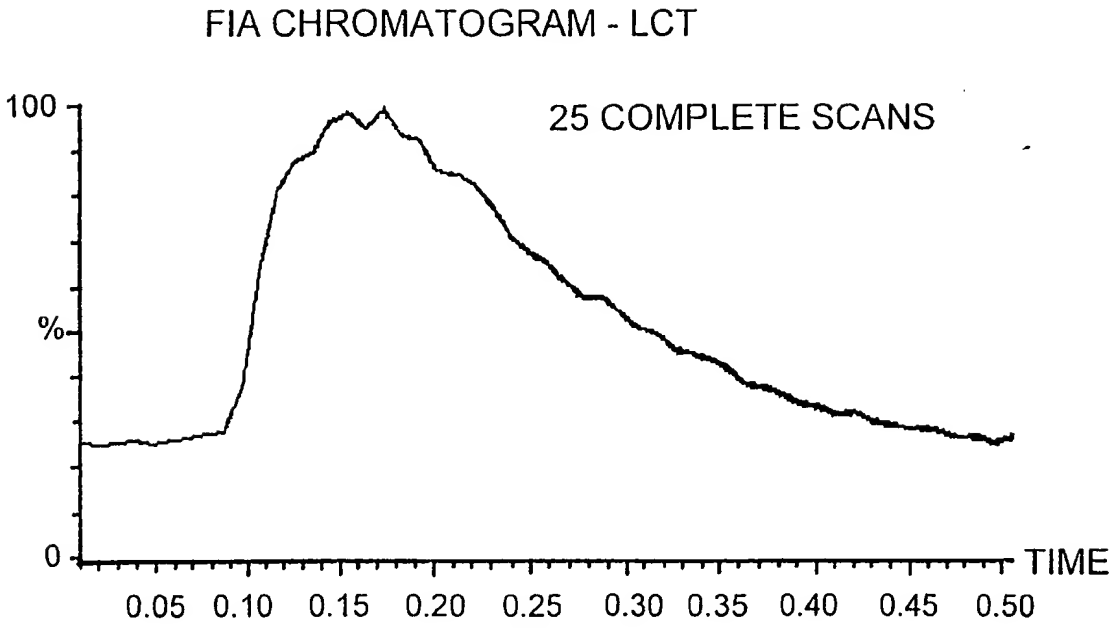
10V
15V
20V
25V
30V
35V
40V

Fig. 5


FIA CHROMATOGRAM - LCT
25 COMPLETE SCANS
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%
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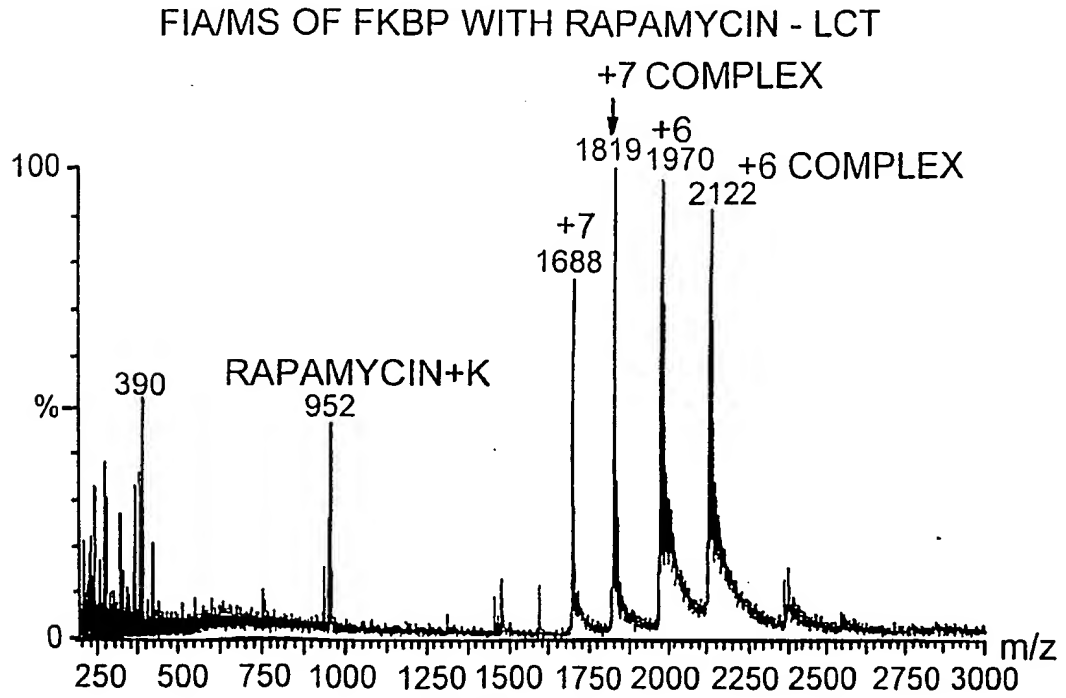
TIME
0.05 0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 0.50

Fig. 6


FIA/MS OF FKBP WITH RAPAMYCIN - LCT
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+6
1819
1970
+6 COMPLEX
2122
+7
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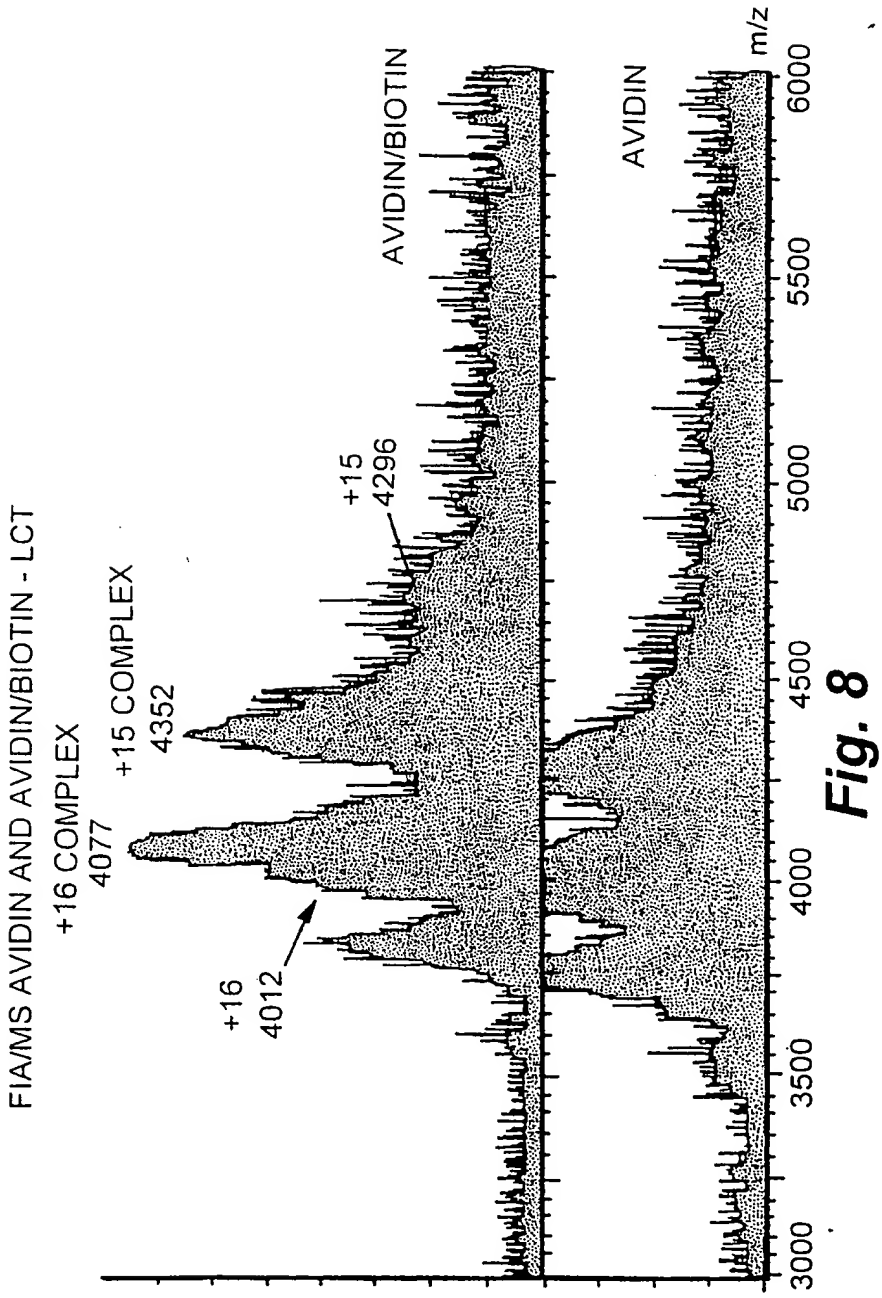
%
0
m/z
250 500 750 1000 1250 1500 1750 2000 2250 2500 2750 3000

Fig. 7


FIA/MS AVIDIN AND AVIDIN/BIOTIN - LCT
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4352
+16
4012
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AVIDIN/BIOTIN
AVIDIN
3000
3500
4000
4500
5000
5500
6000
m/z

Fig. 8

INTERNATIONAL SEARCH REPORT

Internat...al Application No
PCT/US 98/11157

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 H01J49/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 H01J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. CAI: "CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY" JOURNAL OF CHROMATOGRAPHY A , vol. 703, 1995, pages 667-692, XP002076923 see abstract see page 679, right-hand column, line 14 - line 36; figure 1	1,5,8, 11-15, 18,24, 25,27, 28, 32-35,40
A	WO 97 01755 A (PERSEPTIVE BIOSYSTEMS INC) 16 January 1997 see abstract see page 25 see page 41 - page 42	1,24

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 September 1998	22/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hulne, S

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